Media Peternakan, December 2013, pp. 192-196
ISSN 0126-0472 EISSN 2087-4634
Accredited by DGHE No: 66b/DIKTI/Kep/2011

Online version:
http://medpet.journal.ipb.ac.id/
DOI: 10.5398/medpet.2013.36.3.192

Characterization of Bacterial Mannanase for Hydrolyzing Palm Kernel Cake to Produce Manno-oligosaccharides Prebiotics

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(Received 17-04-2012; Reviewed 06-05-2013; Accepted 26-11-2013)

ABSTRACT

Palm kernel cake (PKC) is a promising source of prebiotics, since it contains high amount of β-mannan which can be further hydrolyzed to manno-oligasaccharides (MOS), a prebiotic. Therefore, this research was carried out to analyze the capability of a bacterial isolate (A2 isolates) previously isolated from soils sample from around IPB campus to hydrolyze PKC. Based on 16S-DNA analysis, isolate A2 was identified as *Brevibacillus borstelensis*. Mannanase of A2 isolate had an optimum condition at 90 °C and pH 7. Mannanase activity of crude extracts using Locust Bean Gum (LBG) and PKC as substrates were 0.37U/mL and 0.032U/mL, respectively. However, the most favorable production of oligosaccharides based on the degree of polymerization was obtained after 72-h of incubation with the ratio of substrate:enzyme, 1.2:1, on 1.5% PKC as substrate. The manno-oligosaccharides prebiotic obtained was found to interfere the growth of both lactic acid bacteria (*Lactobacillus casei*) and pathogenic microflora (*Escherichia coli*). *E. coli* apparently could not use this prebiotic as the carbon sources, in contrast to *L. casei*. Substitution of carbon source in medium with prebiotics reduced the capability of *L. casei* to produce organic acids. It is concluded that local A2 isolate (*B. borstelensis*) produces mannanase which can be used to produce prebiotics from PKC.

Key words: Brevibacillus borstelensis, mannanase, Lactobacillus casei, PKC

ABSTRAK

Komponen utama dari bungkil inti sawit (BIS) adalah β-manan yang dapat digunakan dalam produksi prebiotik Manno-oligosakarida (MOS). Penelitian ini bertujuan mengkarakterisasi kemampuan isolat bakteri A2 yang diisolasi dari sampel tanah di sekitar kampus IPB dalam mendegradasi BIS. Berdasarkan analisis 16S-DNA isolat A2 teridentifikasi sebagai *Brevibacillus borstelensis*. Mananase isolat A2 memiliki kondisi optimum pada suhu 90 °C dan pH 7. Aktivitas mananase isolat A2 pada media dengan Locust Bean Gum (LBG) dan BIS berturut turut adalah 0.37U/ml dan 0.032U/ml. Perbandingan substrat:enzyme: 1.2:1, pada substrat BIS 1.5% memberikan derajat polimerisasi terbaik bagi produksi oligosakarida. Prebiotik MOS ini dapat mempengaruhi pertumbuhan *Escherichia coli* patogen dan *Lactobacillus casei*. *E. coli* tidak dapat menggunakan prebiotik ini sebagai sumber karbon dibandingkan *L. casei*. Substitusi sumber karbon dengan prebiotik menurunkan kemampuan *L. casei* dalam memproduksi asam organik. Disimpulkan bahwa isolat lokal A2 (*B. borstelensis*) menghasilkan mananase yang dapat digunakan untuk menghasilkan prebiotik dari BIS.

Kata kunci: Brevibacillus borstelensis, mananase, Lactobacillus casei, PKC

INTRODUCTION

Palm oil processing produces by-products such as palm kernel cake (PKC), which can reach 45%-46% of palm kernel. PKC generally contains approximately 15.4% crude protein and 16.4% crude fiber (Adrizal *et al*., 2011). Approximately 20%-40% of the fiber composition of palm kernel cake is in the form of β–mannans (Yopi *et al*., 2006). This abundant by-product of palm oil industry is used as animal feed. Due to its high content of hemicellulose (mannan and galactomanan) and low essential amino acids contents, the use of palm kernel cake in animal feed is limited to ruminant animals (Yopi *et al*., 2006, Sakamoto & Toyohara, 2009).

The use of antibiotics in the feed has been banned in European Union since 2006. This is due to the con-

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cern of either antibiotic residues or antibiotic-resistant microorganisms in the human body or livestock (especially pathogenic bacteria such as Salmonella, *E. coli* and *Clostridium perfringens*). Prebiotics and probiotics have been used as alternatives to replace the use of antibiotics in animal feed (Reid & Robert, 2002; Samadi, 2002; Urashima & Taufik, 2010). The use of prebiotics can increase the number of beneficial bacteria in the intestine such as *Lactobacillus* and *Bifidobacterium*, therefore suppressing the growth of pathogen and increasing resistance to infection. Mannan can be hydrolyzed into manno-oligosaccharides (MOS), a type of prebiotics. In the previous report, supplementation of the diet with MOS showed a significant reduction of the total population of *E. coli* and *Salmonellae* in the broiler chicken feces (Khanongnuch *et al*., 2006). The balance of intestinal microflora was also related to MOS supplementation. This has beneficial effect in enhancing the growth of desirable microorganisms and thus may stimulate immune system (Shashidhara & Devegowda, 2003). A study was then conducted to evaluate the capability of mannanolytic bacteria to use PKC as the mannan sources to produce manno-oligosaccharide prebiotics. This study was also aimed at characterizing the mannanolytic enzyme.

MATERIALS AND METHODS

Enzyme Characterization

A2 isolate isolated from soils around the IPB campus has been shown to have the ability to use PKC as a carbon source. As the main culture, isolates were grown in a medium containing locust bean gum (LBG) (0.5%), KNO_3 (0.2%), K_2HPO4, (0.1%), $MgSO_4$ (0.05%), NaCl (0.05 %), $FeSO_4$ (0.001%) and $CaCO_3$ (0.3%) for 3 d. LBG is a common medium for producing mannanase. Main cultures were then sub-cultured into 100 ml of LBG liquid medium in 500 mL flask and further incubated in a shaker incubator (120 rpm speed). Crude extract was obtained from each culture by centrifugation at 3000 g for 25 min. The enzyme activity of crude extract was measured by using DNS method (Miller, 1959), with mannose as the standard. The amount of reducing sugars yield was assessed by spectrophotometry analysis (540 nm).

Crude extracts were used as the enzyme source, and further be examined for mannanolytic activity at various pH ranging from 3 to 8.5, and temperature ranging from 30 °C to 90 °C. The ability of enzyme to hydrolyze PKC was tested at its optimum temperature and pH obtained previously. The concentration of PKC tested varied (0.5%, 1.0%, and 1.5%). The dynamic of hydrolyzing activity was measured every 24 h, based on the amount of reducing sugar, total sugar and the degree of polymerization (DP) of the hydrolyzed products.

The Influence of Manno-oligosaccharides Prebiotic on the Growth of Lactic Acid and Pathogenic Bacteria

The effectiveness of manno-oligosaccharides prebiotic produced from PKC hydrolyzation was further tested against bacterial growth. One colony of either lactic acid bacteria or pathogenic bacteria were grown in LB or MRS liquid medium, respectively, and incubated at 37 °C for 24 h. There were six treatment groups, designated as 1: *L. casei* in minimum MRS media (glucose concentration was reduced to one-tenth), 2: *L. casei* in minimum MRS which glucose was substituted with prebiotic, 3: *L. casei* in MRS medium, 4: *E. coli* in minimum LB medium (trypton concentration was reduced to one-tenth), 5: *E. coli* in minimum LB medium substituted with prebiotics (tripton was substituted with prebiotics), 6: *E. coli* in LB media. After incubation, cell's population was calculated in each treatment based on TPC method. Prebiotic which substituted glucose had a DP about 12 and had an equal concentration with glucose. All data were analyzed using descriptive analysis.

Volatile Fatty Acids

The volatile fatty acids (VFA) production of LAB using prebiotics as a substrate was measured using a Chrompack 9002 gas chromatograph. The VFA production indicates the growth of bacteria and its ability to use carbon sources available.

16S-rRNA Gene Analysis

Isolate A2 was identified based on molecular methods. Genomic DNA was isolated using standard CTAB method as described by Sambrook *et al.* (1989). Genomic DNA was then used for PCR amplification by using 16S-rRNA specific primer as follows: 63f (5'-CAG GCC TAA CAC ATG CAA GTC – 3') and 1387r (5'- GGG CGG WTG GTA CAA GGC-3') (Marchesi *et al.*, 1998). DNA amplification was conducted for 30 cycles with following condition: pre denaturation for 2 min at 94 °C, denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s, extension at 75 °C for 1 min. PCR product was used for DNA electrophoresis by using 1 % agarose gel and further visualized by using EtBr staining and UV illumination. DNA amplicon was further lead to DNA sequencing. Homology analysis was done by using Basic Local Alignment Tools (BLASTN) method on GenBank database.

RESULTS AND DISCUSSION

The daily activity of mannanase derived from the crude extract of isolate A2 was tested at pH 7 and 30 ^{0}C using either LBG (Figure 1) or PKC (Figure 2) as the main substrate. The highest mannanase production was achieved on day-6 with activity of 0.37 U/mL and in day-4 with activity of 0.032 U/mL in both LBG and PKC as substrates respectively. LBG consists of galactomannan and galactose, so high enzyme activity indicates the enzymes ability to degrade galactomannan. PKC is a complex medium which consists of β-mannan, xylan, arabinan and cellulose (Oluwafemi, 2009; Sakamoto & Toyohara, 2009). Different ability to degrade mannan substrate has been shown by isolate RA05 which was not able to degrade mannan of sugar palm but it had the activity on PKC (Meryandini *et al*., 2010). Bacillus subtilis WY34 exhibited high activity on galactomannan from

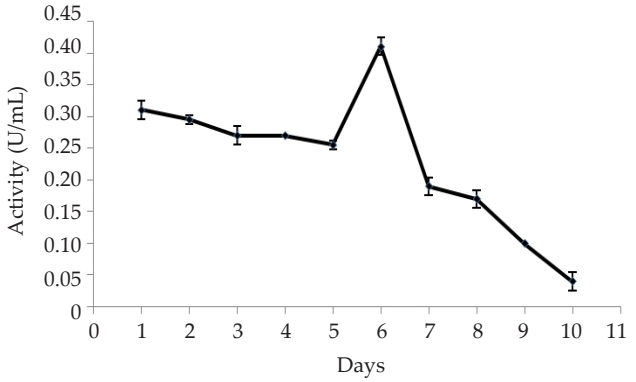

Figure 1. Mannanase activity curve from A2 isolate at pH 7 and 30 °C on LBG 0.5%

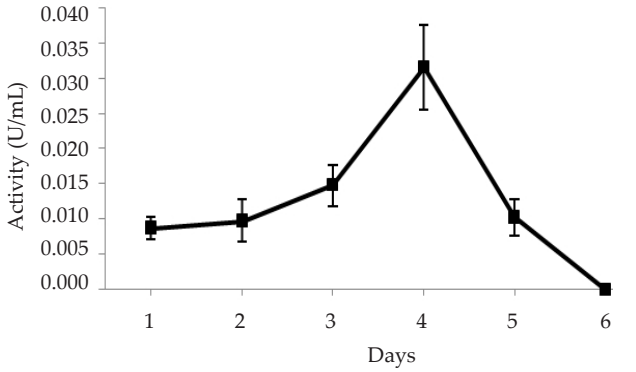

Figure 2. Mannanase activity curve from A2 isolate at pH 7 and 30 °C on PKC 0.5%

LBG (100%), but just 56.8% in Konjac powder, 20.4% in copra mannan and 10.7% in guar gum galactomannan (Jiang *et al*., 2006). The optimum time of mannanase production was then used as the standard for the harvesting time of bacterial cells crude extract in the following mannanase production.

Mannanase production from isolate A2 had the highest activity at 90 °C and pH 7 (0.256 U/mL) when compared to the activity of this enzyme at different pH and temperature (Table 1). Moreover, mannanase of isolate A2 also showed high activity at pH 8 (0.253 U/mL) when measured at 90 °C. Similar thermophilic mannanase enzyme had been isolated from *Thermotoga neapolitana* 5068 that had the optimum temperature at 87 °C (Duffaud *et al*., 1997).

Increase in temperature leads to the increase in thermal energy of the substrate molecule and thus increases the rate of enzyme reactions. Acidic or alkaline pH conditions affect the ionization of the active enzyme that will affect the affinity of the enzyme to the substrate and the enzymatic reaction rate. However, in this study, mannanase of isolate A2 was observed to have lower activity when using PKC as the substrate compared to LBG. It might be due to the differences in chemical content of the PKC and LBG that may affect the enzymatic activity. As previously reported, LBG consists of galactomannan and galactose, while the composition of PKC besides β-mannan are xylan, arabinan and cellulose (Oluwafemi, 2009; Sakamoto & Toyohara, 2009).

Table 1. Mannanase activity on 0.5% LBG medium at different pH and temperature

Temperature (°C)	pH	Activity (U/mL)
30	3	0.000
	4	0.000
	5	0.131
	6	0.084
	7	0.058
	8	0.131
40	3	0.000
	4	0.000
	5	0.132
	6	0.021
	7	0.124
	8	0.148
50	3	0.000
	4	0.000
	5	0.000
	6	0.106
	7	0.112
	8	0.117
60	3	0.000
	4	0.000
	5	0.000
	6	0.068
	7	0.131
	8	0.033
70	3	0.000
	4	0.000
	5	0.000
	6	0.000
	7	0.073
	8	0.033
80	3	0.000
	4	0.000
	5	0.000
	6	0.059
	7	0.124
	8	0.063
90	3	0.000
	4	0.000
	5	0.091
	6	0.227
	7	0.256
	8	0.253

The best degree of polymerization (DP) was obtained after 72-h of incubation, with the ratio of substrate (1.5% PKC) : enzyme was 1.2: 1 Table 2). This

condition was further used for optimum production of oligosaccharides.

One way to test the prebiotics produced from PKC was its ability to enhance the growth of BAL and to depress the growth of *E. coli*. The ability of *E. coli* to use prebiotics as the carbon and energy sources was analyzed for 48 h. In the first 24 h *E. coli* was still able to grow in the minimum LB media without the prebiotic or prebiotics substitution. It might be due to the ability of *E. coli* to continue their metabolism after grown on LB medium. Prebiotics used as carbon source was calculated through degree of polymerization so it could consist of small fraction of short chains of monosaccharides or oligosaccharides that were easily digested by *E. coli*. Numbers of cells in minimum LB medium (4×10^7 cells/mL) was lower than that in LB medium (2.3×10^{15} cells/mL). Interestingly, substitution of prebiotics in minimum LB medium increased cells viability, whereas population of *E. coli* in LB medium containing prebiotics was approximately 3.5×10^{11} cells/mL. Similarly, *L. casei* was also found to be more viable in minimum MRS medium supplemented with prebiotics (1.1×10^8 cells/mL) compared to without prebiotics treatment (2×10^7 cells/mL). However, these number of cell's population were still lower compared to the growth of *L. casei* in its rich MRS medium (1.08×10^{14} cells/mL). In addition, pH value of the culture in rich MRS medium was lower (pH= 3.55) than those cultures in minimum MRS medium (6.39 and 6.55) with and without prebiotics substitutions, respectively.

To further study the growth of *E. coli* at the next 24 h in minimum medium containing prebiotics, *E. coli* was sub-cultured from minimum LB medium to a new similar medium. In contrast to its earlier 24 h studies, the growth of *E. coli* in minimum LB medium containing prebiotics (4×10^7 cells/mL) was lower than those without prebiotics treatment (3×10^9 cells/mL). After the first 24 h of growth *E. coli* was not able to maintain their growth because they could not use prebiotics compared to the growth of *E. coli* on LB minimum which still contained a low level of tripton (1%). Thus, suggesting that *E. coli* could not use prebiotics as the carbon sources. In the case of *L. casei*, co-inoculation of culture from minimum MRS medium containing prebiotics to the same medium also affected bacterial cell viability. Contrary to the response of *E. coli*, *L. casei* was observed to have better growth in minimum MRS medium containing prebiotics (1.4×10^{11} cells/mL) compared to those without prebiotics treatment (3.2×10^{10} cells/mL). These results indicated that *L. casei* could utilize prebiotics as the carbon source. It has been previously reported that *L. casei* can produce several types of enzymes that enable the bacteria to utilize oligosaccharides such as α-galactosidase, α--mannosidase, β-glucosidase, α-glucosidase, and β-galactosidase (Arora *et al.*, 1990).

The growth of *L. casei* in the first 24 h was lower compared to the growth of *E. coli* as shown in total colony formed. However, in the next 24 h, *L. casei* seemed to grow faster than *E. coli*. *In vitro* test using XOS as a prebiotics for accelerating the growth of Bifidobacteria was also done by Zeng *et al*. (2007) and Su *et al*. (2007). Baurhoo *et al.* (2007) and Kim *et al.* (2011) also reported that provision of prebiotics increased the number of probiotic bacteria. Moreover, Khanongnuch *et al.* (2006) and Baurhoo *et al.* 2009 also reported that population of *E. coli* and salmonellae in chicken faeces decreased significantly due to the effect of MOS. Therefore, the effect of stimulating the growth of *L. casei* and suppressing the growth of *E. coli* can be tested *in vivo* by prebiotic administration on an ongoing basis so that its influence to intestinal microflora balance can be determined.

Carbohydrate fermentation by *L. casei* produced metabolites of organic acids such as short chain fatty acids. Analysis of VFA was particularly aimed to determine the amount of short-chain fatty acids produced by *L. casei* on MRS medium with prebiotics as well as on MRS media.

Total short chain fatty acids production in MRS culture (22.72 mmol) was higher than those in the MRS culture with prebiotics substitution (12.06 mmol). These results were also supported with pH measurement data, in which the pH of culture in MRS medium with prebiotics was higher (6.39) than the pH in MRS medium (3.55). In the MRS medium the BAL fermented glucose (monomer) and produced organic acid whereas in the MRS medium with prebiotics, the glucose was substituted with prebiotics (polymer). Low concentration of short chain fatty acids of culture grown in MRS medium with prebiotics (Table 3) was probably caused by modification or changes in certain metabolism pathways since MOS was used as the only carbon source replacing glucose. However, *L. casei* was still able to grow in this medium. In addition, the degree of polymerization value (DP) of the prebiotic MOS ranged from 8 to10, which was still higher than the commonly used prebiotics, which is about 3-7 with a purity of 95% (Pan *et al.*, 2009). Hence, it would affect the capability of probiotic bacteria to do

Table 2. Degree of polymerization (DP) of PKC using mannanase A2 (substrate : enzyme= 1.2 : 1)

PKC concentration	Hour	Total sugar	Reducing sugar	DP
0.50%	1	3.786	0.264	14.357
	24	3.851	0.370	10.417
	48	2.113	0.186	11.381
	72	7.629	0.519	14.709
	96	9.041	0.488	18.540
1.00%	1	3.851	0.304	12.680
	24	5.013	0.614	8.168
	48	5.197	0.318	16.360
	72	7.839	0.583	13.453
	96	10.703	0.591	18.120
1.50%	1	6.229	0.324	19.244
	24	9.910	0.672	14.755
	48	5.425	0.451	12.038
	72	7.426	0.679	10.942
	96	8.064	0.691	11.676

Table 3. Volatile fatty acids (VFA) contents of MRS and MRS added prebiotics

Sample	VFA (mM)					
	Acetic acid	Propionate acid	Iso butirate	n-butirate	Iso valerat	n-valerat
MRS	113.54	5.06	1.48	1.89	0.75	0.0
MRS with prebiotics	6.13	2.94	0.72	1.78	0.52	0.0

fermentation which further lowers the amount of fermentation products, including organic acids.

Based on homology analysis, isolate A2 possessed high similarity with *Brevibacillus borstelensis* (100%). *Brevibacterium borstelensis* was equivalent with *Bacillus borstelensis* (Shida *et al*., 1996). *B. borstelensis* was also known as keratinase and lipase producing bacteria (Hoq *et al*., 2005; Panda *et al*., 2011).

CONCLUSION

Local A2 isolate (*B. borstelensis*) produces mannanase which can be used to produce prebiotics from palm kernel cake (PKC). The manno-oligosaccharides prebiotics can enhance the growth of *L. casei* and suppress *E. coli* growth.

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